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MIDGET cooperates with COP1 and SPA1 to repress flowering in *Arabidopsis thaliana*

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The life cycle of plants is strictly regulated by light, which directly influences the initiation of developmental programs such as photomorphogenesis of seedlings and induction of flowering. When environmental conditions are unsuitable, both processes are actively repressed by the action of COP1/SPA protein complexes which participate in ubiquitylation and subsequent degradation of transcription factors. We have shown recently that MIDGET (MID), a regulator of the TOPOISOMERASE VI complex, physically interacts with COP1 and is required for its function as suppressor of photomorphogenesis. Here we show that in *Arabidopsis thaliana*, the MID protein similarly plays a role in COP1/SPA1-controlled repression of flowering under short-day conditions.

At temperate latitudes with its seasonal climates, the precise regulation of flowering time is essential for plants to allow seed development in favorable environmental conditions to achieve reproductive success.[1] In the facultative long-day plant *Arabidopsis thaliana*, seed development is restricted to the summer season. Flowering is suppressed under short-day conditions, and de-repression requires both, a period of low temperature (vernalization) and daylight of sufficient length (photoperiod).[2]

Flower transition in *A. thaliana* is controlled by FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF), which in turn are regulated by CONSTANS (CO), a B-box transcription factor.[3-6] Furthermore, expression of FT and similarly expression of its negative regulator FLOWERING LOCUS C (FLC) apparently are regulated by chromatin modification.[7] The expression of CO is stimulated by light and dependent on the circadian clock.[1] Moreover, CO protein accumulation is controlled by an ubiquitin ligase complex including COP1 and SPA proteins, facilitating ubiquitylation-dependent proteasomal degradation.[8,9] SPA1 interacts with COP1 and it enhances in vitro the E3 ubiquitin ligase activity of COP1 toward LONG AFTER FAR-RED LIGHT1 (LAF1), a transcription factor of the phyA activated signaling pathway.[10,11]

COP1/SPA protein complexes in turn are negatively regulated by blue light-induced interaction with cry1 and cry2[12,13] Additionally, phyA, phyB and cry1 reduce the nuclear abundance of COP1 in response to light that is needed for COP1 complexes` function.[14,15] According to the external coincidence model, under long-day conditions CO mRNA accumulation and translation coincide with light-dependent repression by cry2 of the negative regulators COP1/SPA thereby stabilizing CO activity to promote flowering.[1,12]

In a similar way COP1/SPA complexes are involved in the regulation of light-dependent development of seedlings. In the absence of light, the COP1/SPA-dependent ubiquitin ligase activity leads to degradation of photomorphogenesis-promoting transcription factors. Seedlings undergo skotomorphogenic development characterized by rapid hypocotyl elongation concomitant with several rounds of endoreduplication in elongating cells. Endoreduplication in *A. thaliana* is controlled by the TOPOVI complex and its regulators RHL1 and MIDGET.[16-19] Recently we have proven a direct physical interaction of MID with the N-terminus of COP1 in vivo, and we have shown that MID is an essential regulator of COP1 function required for both, repression of photomorphogenesis and for hypocotyl elongation as well as endoreduplication during skotomorphogensis.[20]

Because COP1, SPA1 and MID genes are expressed at later stages of development as well, we hypothesized that such regulation of the COP1/SPA complex is not restricted to seedling stage. Therefore, we investigated *mid* mutants with respect to flowering time. The flowering time was determined by counting the number of rosette leaves when the first bud was visible. Under long-day conditions, wild-type *A. thaliana* Col-0 plants initiated the first bud after 11 rosette leaves have been developed, while the *mid-1* mutant starts transition to flowering earlier, with only 7–9 leaves developed (**Fig. 1A**). The stronger *mid* mutant allele (*mid-2*) and similarly the *topo VI* mutants *hyp6* and *rhl2* exhibit an even stronger early flowering phenotype. The first bud was detectable already after the development of on average 5 leaves (**Fig. 1A**).

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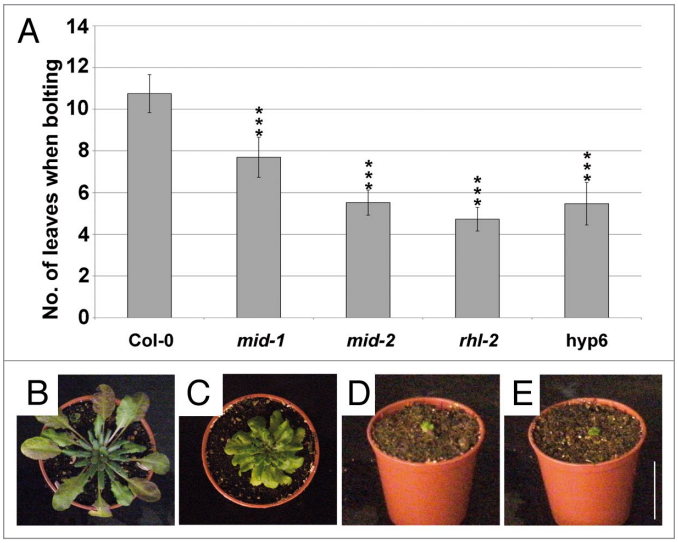

Figure 1. TOPOVI mutants flower earlier under LD conditions and strong TOPOVI mutants are lethal under SD conditions. (**A**) After stratification for 4 d at 4 °C, plants were grown in the greenhouse under long day (LD) conditions (16 h light, 8 h darkness) at 21 °C. The number of true leaves was determined when the first bud was visible. All mutants flowered significantly earlier (***: P < 0.001) than the wild-type. The number of analyzed plants was 203 (Col-0), 175 (*mid-1*), 21 (*mid-2*), 81 (*rhl2*), and 34 (*hyp6*). Please note that due to seed limitations only one replicate could be analyzed for *mid-2*, *rhl2* and *hyp6* as compared with 3 replicates for Col-0 and *mid-1*. (**B–E**) Phenotype of 87-d-old plants grown in a plant chamber under short day (SD) conditions (8h light, 16h darkness) at 21 °C.(**B**) Col-0, (**C**) *mid-1*, (**D**) *mid-2*, (**E**) *rhl2*. All *hyp6* plants died earlier. The *mid-2* and *rhl2* plants on the right died before flowering. Flowering at SD of *mid-1* is shown in **Figure 2**. Even at enhanced light intensities neither *hyp6*, *mid-2* nor *rhl2* could be analyzed under SD conditions. Light intensity in this experiment: 31–46 µmol*m-2*s-1 (Osram Cool White L58W/21–840 Lumilux Plus Eco and Natura de Luxe L58W/76). Bar for (**B–E**) equals 3 cm.

These results indicate that in the *mid* and *topoVI* mutants either the signaling pathway maintaining repression of flowering is compromised, or the chromosomal integrity is affected such that the epigenetic control of flowering time[7] is altered.

Under short-day conditions only the weak *mid-1* mutant could be analyzed (see below), because strong mid mutants and similarly the *topoVI* loss-of-function mutants *rhl2* and *hyp6* did not develop beyond the vegetative growth phase and eventually died without transition to the reproductive phase (**Fig. 1B–E**).

cop1 mutants flower slightly earlier under LD conditions and much earlier in SDs compared with the wild-type, being almost insensitive to the different day lengths.[9,21] In contrast, *spa1* mutants apparently flower earlier only in SDs and not in LDs. *SPA3* together with *SPA4* act redundantly with *SPA1* but are not sufficient for normal flowering under SD conditions. As *SPA1* is sufficient and necessary for normal flowering in SDs,[8] we selected the *spa1* mutant for double mutant analysis. Analysis of double mutants of *mid-1*[18] with *cop1-4*[21] and *spa1-100*,[22] respectively, revealed that, despite the ability of MID to physically interact with COP1, the early flowering phenotype under long day conditions of the *mid* and *topoVI* mutants might not be connected to COP1/SPA function. Both *mid-1* and *cop1-4* as single mutants lead to somewhat earlier flowering with on average 9 and 10 rosette leaves developed, respectively, as compared with 11 leaves at the time of flowering in the wild-type. The *spa1-100* mutation does not result in an early flowering phenotype under long-day conditions, but rather exhibited a small but significant delay in flowering. The double mutant *mid-1cop1-4* flowered earlier than the single mutants (at 7.5 rosette leaves on average). Together with the *topoVI* single mutant phenotype, this enhanced phenotype can be explained by mere additive effects and may not be interpreted as indication of genetic interaction. Similarly there is no indication of genetic interaction of *mid-1* with *spa1-100* under long day conditions. The early flowering phenotype of the double mutant does not differ from the *mid-1* single mutant phenotype (**Fig. 2B**). Under long day conditions, the observed early flowering phenotype of the *mid* and *topoVI* mutants, therefore is probably mechanistically unrelated to COP1/SPA1 function and may be related to their function in chromatin remodeling or in maintaining transcriptional silencing[15] of flowering genes under chromatin regulation.[7]

Under short day conditions, however, we observe synergetic enhanced phenotypes indicative of genetic interaction of the *mid* gene with both, *cop1* and *spa1*, respectively, in controlling flowering time. The single mutation *mid-1* does not lead to early flowering under short-day conditions. In fact, we observe to some degree delayed flowering of the *mid-1* mutant (at 60 rosette leaves on average, as compared with 54 rosette leaves in the wild-type). The *cop1-4* mutant on the other hand, consistent with reports in the literature,[9] showed an extremely early flowering phenotype, producing > 40 leaves fewer than wild-type plants before flowering. Even this strong phenotype, however, is significantly enhanced in the *mid-1cop1-4* double mutant (**Fig. 2C**). A similar result, although to a lesser degree, is obtained from the analysis of *mid-1* and *spa1-100* single and double mutants. The *spa1-100* mutant flowers somewhat earlier than the wild-type plants (50 vs. 55 rosette leaves; **Fig 2D**). The double mutant *mid-1spa1-100*, however, shows a quite pronounced early flowering phenotype with more than 20 leaves fewer than wild-type at the time of flowering (**Fig. 2D**).

Due to our observation that the mid-1 single mutant flowers even slightly later than the wt under SD conditions, we can exclude an effect of plant size on flowering, indicating that the mutations indeed affect light signaling.

Our results indicate genetic interaction of the mid gene with both cop1 and spa1 genes, and, taken together with the proven ability of MID to form a protein complex with COP1 in vivo,[20] strongly suggest that MID has a function in COP1/SPA-dependent repression of flowering under short-day conditions.

What role could MID possibly play in this context? We have shown previously that in the context of photomorphogenetic development of *A. thaliana* seedlings the presence of a functional MID protein is required to stabilize the COP1 protein.[20] With respect to the control of flowering time, this stabilizing function may similarly play a role, although to a somewhat lesser degree: The mid-1 mutation alone apparently is not sufficient to significantly impair COP1 function as a repressor of flower transition,

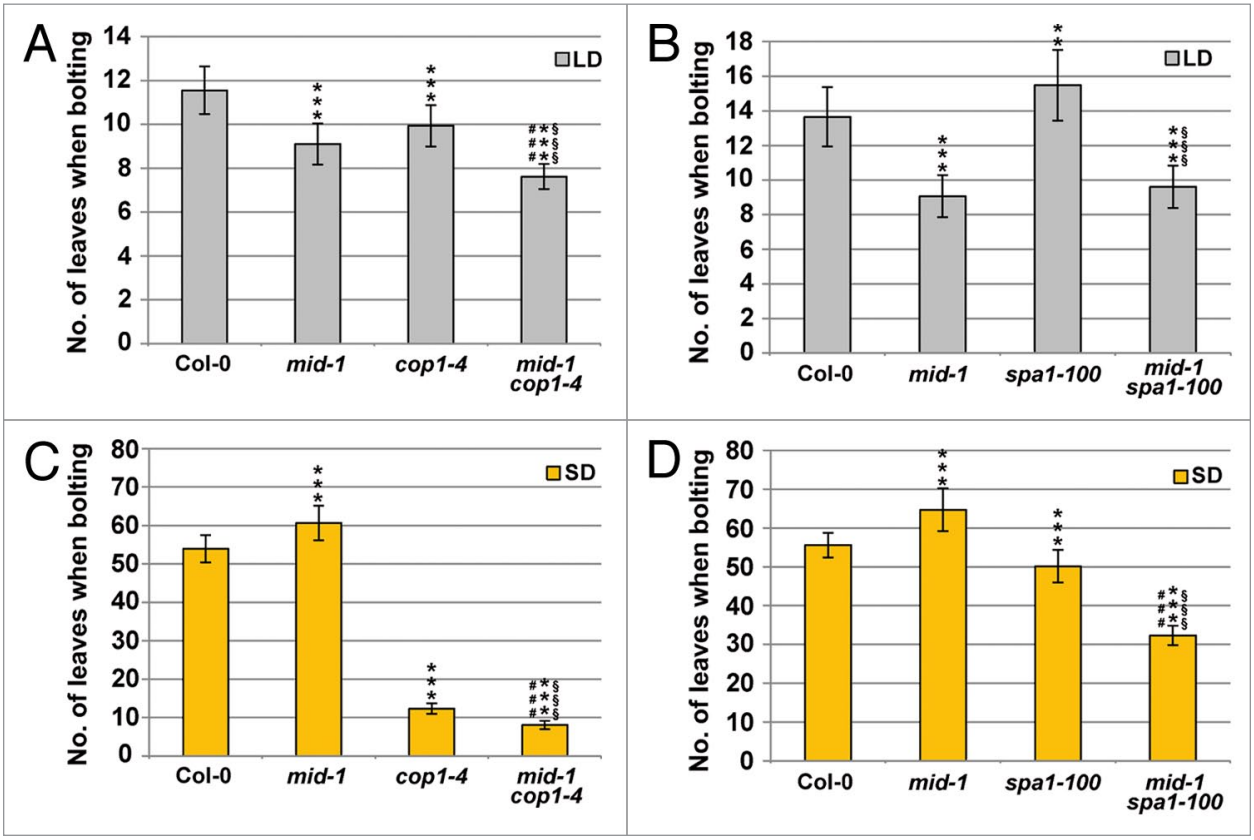

Figure 2. MIDGET interacts genetically with COP1 and SPA1 in flowering time determination. After stratification for 4 d at 4 °C, plants were grown in a plant chamber (120–170 µmol* m-2*s-1, Osram L58W/840 Lumilux Coolwhite) under long day (LD; 16 h light, 8 h darkness; [**A and B**]) or short day (SD; 8 h light, 16 h darkness; [**C and D**]) at 21 °C. The number of true leaves was determined when the first bud was visible. Flowering significantly different from the wild-type is indicated by *, compared with *mid-1* by # and to *cop1-4* or *spa1-100*, respectively, by § (**: $P < 0.01$; ***: $P < 0.001$). Statistic analysis was done as described previously.[20]

because the *mid-1* mutant does not have an early flowering phenotype under short-day conditions. However, the *mid-1* mutation in the *spa1-100* mutant background does have a significant impact on flowering time, probably by further de-stabilizing the COP1 protein, which already is impaired by the lack of SPA1, such that it no longer can fulfill its function completely.

With regard to the impact on the control of flowering time under long-day conditions it remains to be investigated whether MID as a regulator of the TOPOVI complex has an impact directly on the signaling components involved in control of flowering time, or whether more general aspects of the MID/TOPOVI complex such as chromatin organization, control of endoreduplication and/or regulation of transcriptional silencing[16,18] play a role in this context. Our mutant analysis proves that MID/COP1 and MID/SPA1 and thereby presumably MID and COP1/SPA1 act together to prevent the plant from proceeding to reproductive development under unfavorable SD conditions.

It is tempting to speculate that by ensuring the simultaneous presence of the MID/TOPOVI- and COP1/SPA1-complex genome integrity and proper developmental timing is guaranteed.

In the future, a detailed analysis of MID- and TOPOVI-dependent CO/FT gene expression and protein abundance, as well as a focus on COP1/SPA1 (sub) nuclear accumulation under various conditions will be instrumental to unravel the nature of the MID-dependent regulation of flowering in *A. thaliana*.

Taken together, we have shown that MID is a co-factor of COP1 function, not only in facilitating skotomorphogenetic development of *A. thaliana* seedlings in the dark, but also in connection with COP1 function as a repressor of flowering. This function is evident under short day conditions and mainly in the SPA1 mutant background. Here, loss-of-function of MID leads to early flowering, consistent with the idea that MID is necessary to stabilize COP1.[20]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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